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Comparison of morphological and molecular genetic sex-typing on mediaeval human skeletal remains☆

Christiane Maria Bauer [a,1], Harald Niederstätter [a,1], George McGlynn [b], Harald Stadler [c], Walther Parson [a,d,*]

[a] Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria
[b] Bavarian State Collection for Anthropology and Palaeoanatomy Munich, Munich, Germany
[c] Institute of Archaeologies, University of Innsbruck, Innsbruck, Austria
[d] Penn State Eberly College of Science, University Park, PA, USA

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ABSTRACT

Archaeological excavations conducted at an early mediaeval cemetery in Volders (Tyrol, Austria) produced 141 complete skeletal remains dated between the 5th/6th and 12th/13th centuries. These skeletons represent one of the largest historical series of human remains ever discovered in the East Alpine region. Little historical information is available for this region and time period. The good state of preservation of these bioarchaeological finds offered the opportunity of performing molecular genetic investigations. Adequate DNA extraction methods were tested in the attempt to obtain as high DNA yields as possible for further analyses. Molecular genetic sex-typing using a dedicated PCR multiplex ("Genderplex") gave interpretable results in 88 remains, 78 of which had previously been sexed based on morphological features. We observed a discrepancy in sex determination between the two methods in 21 cases. An unbiased follow-up morphological examination of these finds showed congruence with the DNA results in all but five samples.

1. Introduction

The small Austrian village of Volders is located in the lower Tyrolean Inn Valley. This region had already been settled between the late Neolithic to Early Bronze Age and the Roman era, and was later colonized by Rhaetian and German speaking tribes, including the Bavarians. During the Roman era Volders acted as an important station along the thoroughfare connecting the Italian peninsula with the North. In the end of the 6th century Bavarians settled in the region and lived side by side in admixture with the local inhabitants and the Romans [1].

The reconstruction of early mediaeval rural populations has usually been limited to archaeological, anthropological, and historical research. In Volders, however, excavations carried out by municipal archaeologists revealed the presence of an early mediaeval cemetery (Fig. S1, [1], Alexander Zanesco, Institute of Archaeologies, Innsbruck) that represents one of the largest series of historical human remains found in Tyrol. In an area covering approximately 140 m^2 and two main allocation layers, a total of 153 graves were documented containing a total of 141 nearly complete skeletons. These were subsequently examined and dated between the 5th/6th and 12th/13th centuries [1].

This skeletal assemblage is exceptional for the Alpine region both with respect to the number of individuals as well as to the state of skeletal preservation (Fig. S2a, b, [2], Alexander Zanesco, Institute of Archaeologies, Innsbruck). The cemetery is located close to the upper rim of the ancient bank of the Inn River, which may be one reason for the good state of preservation of some of the burials. The geological layers underneath the burials, which for the most part were interred in soil, are comprised of loose riverbed gravel and stones. This allowed for rapid drainage of rainwater and subsequent better bone preservation. Skeletons that were buried deeper and covered in this gravel were actually preserved less well, since the stones exerted a grinding action on the bones. The archaeological survey brought interesting findings such as different directional orientations of the burials (mainly east–west with a few north–south, Fig. S1, Alexander Zanesco, Institute of Archaeologies, Innsbruck), the presence of stone encirclements around some of the graves and clothing accessories (iron belt

* Corresponding author at: Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria. Tel.: +43 512 9003 70640; fax: +43 512 9003 73640.
E-mail address: walther.parson@i-med.ac.at (W. Parson).

[1] These authors contributed equally to this work.

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buckles with silver inlay, knives, metal belt strap ends and combs) which are typical of the late Roman and the early mediaeval periods [1]. The recovered remains appeared to be suitable for molecular genetic analyses through which – by interdisciplinary collaboration – more light may be shed on the make-up of this past population. Prior to DNA extraction initial experiments were conducted to determine the most suitable DNA extraction method. After extraction DNA was quantified using a real-time PCR approach and sex-typed with a previously described, home-made PCR multiplex ("Genderplex") [3]. The results were compared to morphological sex typing and the findings are discussed highlighting the advantages and limitations of the applied methods.

2. Materials and methods

2.1. Samples

Following completion of the archaeological and anthropological investigations the skeletal remains were stored at room temperature in carton boxes at the Museum of Industry and Prehistory in the neighbouring town of Wattens for about 10 years. A total of 305 samples including femora and humeri as well as teeth (preferentially molars) were chosen for molecular genetic investigations, as those were the most promising of the available tissues according to our experience. Small pieces (ca. 2 cm × 1 cm × 1 cm) of each bone specimen were excised with a bone saw and molars were extracted using forceps. Buccal swabs were collected under written consent from a total of 81 individuals who handled the remains during the excavation process and the anthropological work (n = 22). The associated DNA profiles were added to those of the entire laboratory staff (n = 59) to build a contamination elimination dataset.

2.2. DNA extraction

2.2.1. Physical and chemical sample pre-treatment

The mechanical and chemical processing of the samples was performed with the necessary care required for challenging samples [4,5].

A total of 194 samples were taken from the 141 skeletons (Table 1) and subjected to mechanical surface cleaning with sterile scalpel blades. Samples were then bathed in sodium hypochlorite (≥4% active chlorine, Sigma Aldrich, St. Louis, MO, USA) at room temperature for 15 min, washed in purified water (DNA/RNA free), rinsed in absolute ethanol for 5 min and UV irradiated for 10 min (λ = 254 nm). Samples were dried in a closed laminar flow cabinet over night and then powdered using a vibrating ball mill (Laarmann Group BV, Roermond, The Netherlands).

2.2.2. Demineralization and DNA extraction

About 100–150 mg bone powder were fully demineralized and lysed in 7 ml lysis buffer (500 mM Na_2EDTA (pH 8.0), 0.5% *N*-lauroylsarcosine sodium salt (both Sigma Aldrich), 250 μl 20 mg/ml proteinase K (Roche, Basel, CH)) in a rotary shaker at 56 °C overnight as detailed in [6]. For the DNA extraction from the demineralization/lysis supernatants three published protocols either directly using the silica columns and buffer set included in the QIAamp DNA Blood Maxi kit (Qiagen, Hilden, Germany; SC method) [7], phenol/chloroform/isoamyl-alcohol (PCI) [8], or a spin filter method (SF) [9] were compared in a series of initial experiments on three specimens. Both the PCI and the SF protocol featured a final purification step on a silica matrix using the MinElute PCR Purification kit (Qiagen) according to the manufacturer's recommendations with two additional washing steps.

In the modified SF protocol the demineralization/lysis supernatants (7 ml) were diluted with an equal volume of water (DNA/RNA free) before concentrating them in centrifugal filter units (Amicon Ultra 15, 30K MWCO, Millipore, Billerica, MA, USA). This additional step in the protocol appeared to attenuate the risk of filter-clogging and reduced the concentrations of the lysis buffer constituents in the retenate recovered from the spin filters. All further steps of the SF protocol were performed as described in [9]. The final volume of DNA extracts was 50–75 μl for all approaches. Reference samples on buccal swabs were DNA extracted using a Chelex (Bio-Rad, Hercules, CA, USA) method according to [10].

Table 1
Number of extractions performed per individual skeleton (including pre-studies and repeat extractions).

Number of DNA extractions (n = 194) performed on individuals (n = 141)	
Single	96
Double	38
Triple	6
Quadruple	1

2.3. Real-time PCR DNA quantification

The quantity of total genomic (g)DNA was determined using a real-time PCR approach targeting human specific AluYb8 sequences according to Walker and colleagues [11] with modifications. To monitor potential inhibition we co-amplified a spiked in vitro mutagenized and cloned part of the human retinoblastoma susceptibility protein 1 (RB1) gene as internal PCR control ($pRB1_{IPC}$) [12]. Amplifications were performed in 20 μl reactions containing 1× TaqMan Universal PCR Master Mix (Life Technologies (LT), Carlsbad, CA, USA), 5 μg non-acetylated bovine serum albumin (Sigma Aldrich), 400 nM each of the two AluYb8 amplification primers (F: CTTGCAGTGAGCCGAGATT, R: GAGACGGAGTCTCGCTCTGTC, [11]), 200 nM AluYb8 probe (FAM-ACTGCAGTCCGCAGTCCGGCCT-NFQ/MGB, [11]), 900 nM RB1-157-F (CCAGAAAATAAATCAGATGGTATGTAACA, [12,13]), 900 nM RB1-IPC-R (TCGTTTCGGAGCGTTGGTTAG, [12]), 200 nM RB1 hybridization probe (VIC-CAGCACTTCTTTTGAGCAC-NFQ/MGB, [12]), and 20,000 $pRB1_{IPC}$ plasmids [12]. Kinetic PCR was conducted in 96-well polypropylene PCR plates on a 7500 Fast Real-Time PCR System (LT) using the "7500 standard setting" for the speed of all temperature transitions. The thermal cycling protocol comprised of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. The 7500 Fast System Sequence Detection Software (v1.4, Applied Biosystems/LT) was used for signal read-out during the annealing/extension step and data analysis. Calibration curves covered a gDNA input range of 15 ng–254 fg per 20 μl reaction. The human genome accommodates multiple AluYb8 repeats, which explains the high sensitivity of the quantification assay used here. For instance, by searching the publicly available databases Gibbons and colleagues [14] constructed a library of 2201 AluYb8 repeats found in the human genome, and by using this information we identified 1072 templates with perfectly matching primer and probe binding sites. The deduced minimum and maximum amplicon lengths were 71 bp (n = 1068) and 94 bp. Based on the sequence information in the AluYb8 repeat library we also designed an alternative TaqMan probe (FAM-CGGACTGCGGACTGCA-NFQ/MGB) and an alternative forward primer (AluYb8-F_2: GGGTGGATCATGAGGTCAGGA). For the AluYb8-F_2 ↔ AluYb8-R plus alternative probe trio 1075 perfectly matching template sequences were obtained (amplicon size range: 196–261 bp, 921 × 235 bp). This "AluYb8 long amplicon" approach (using the 71 bp amplicon PCR conditions but 250 nM each primer, 200 nM alternative probe, and extra AmpliTaq Gold DNA polymerase (2 units, LT) was successfully tested in initial experiments. The linear dynamic range was comparable to that obtained

for the 71 bp amplicon, albeit at a markedly lower apparent single cycle PCR efficiency (data not shown). The AluYb8 long amplicon approach did not allow for the co-amplification of the pRB1-IPC, but parallel use of the two AluYb8 assays would allow for DNA quantification, inhibitor detection and assessment of the DNA fragmentation. However, to save precious sample we refrained from using the AluYb8 long amplicon approach in this study.

2.4. Sex-typing

Morphological sex identification was conducted by assessing defined metric criteria of the pelvis bone according to [1,15]. Molecular genetic sex-typing was performed on the DNA extracts using the Genderplex PCR system that co-amplified two independent regions of the amelogenin gene, a fragment of the sex determining region on the Y-chromosome (SRY) and the four X-short tandem repeat (STR) markers DXS7424, DXS8378, DXS6803, and GATA172D05 [3].

2.5. Autosomal STR typing of controls

All extraction blanks were subjected to autosomal STR typing using maximum volume of extracted DNA (10 μl) and the PowerPlex ESX 17 or ESI 17 Systems (Promega, Madison, WI, USA) following the manufacturer's recommendations except for extending the PCR to 34 cycles.

3. Results and discussion

3.1. Initial studies on extraction methods

In a first pre-study the performance of three published DNA extraction protocols was compared using three skeletal remains. Two of these samples came from the Volders cemetery. One displayed a generally good condition (V1) and the other one showed signs of severe decomposition (V2). The third sample was provided by the Institute of Archaeologies, University of Innsbruck and was recovered during an archaeological excavation from Kals (Großberg, Tyrol, Austria) performed in 2006. This generally well-preserved sample originated from an inhumation burial at about 1400 m sea level and was dated between the 6th/7th centuries. The DNA yields were comparable for samples V1 and V2 among the three extraction methods, only with the third sample (Kals) SC and PCI, gave higher quantities than SF (Table 2). Due to the low DNA quality and quantities to be expected and the relatively complex laboratory procedures (e.g., multiple handling steps) contamination had to be considered a serious issue and was observed for the SC method. The PCI method produced the second highest DNA yields and did not exhibit signs of detectable contamination. However, this method is time intensive and necessitates the use of toxic reagents. For all three methods co-extraction of PCR inhibitors could be ruled out by the results of the real-time PCR quantification assay including an internal amplification positive control system (data not shown).

We decided to modify the lysis component of the SF method and to repeat the test in comparison to the PCI method, while the SC method was discontinued due to its susceptibility to contamination. In a second pre-study, tooth and bone samples collected from three unknown museum specimens were used. The PCI and the modified SF methods showed comparable DNA yields for all three samples with a general trend towards a slightly better performance of the modified SF method (with the exception of the bone sample of Ind.1; Table 2). Low DNA amounts in the blanks were observed for both methods this time. The blanks of both pre-studies were analyzed with autosomal STRs and yielded sparse drop-in alleles and in the case of the SC blank in pre-study 1 a female partial profile (Table 3). Due to the simpler handling protocol and the absence of hazardous reagents the modified SF method was further applied to all remains investigated from the Volders cemetery. The PCI method was used as a follow-up option for those remains that did not yield sufficient DNA for downstream analyses (threshold for repeat extraction: <2 pg/μl; Table 3, Table 4).

3.2. Main study: DNA yield

The human remains recovered from the mediaeval cemetery were extracted in 18 batches (PCI and SF extractions) of 4–13 samples (Table 3). With the exception of the second batch the extraction blanks showed negligible DNA concentrations (between 0.01 and 0.25 pg/μl), which did not result in autosomal STR profiles except of infrequent drop-in alleles with low relative fluorescence unit (RFU) values. The blank from the second batch (1.6 pg/μl) yielded a low (150 RFU) male partial STR profile. This particular profile differed from both those of the other samples in the affected batch and from the profiles in the contamination elimination database. Therefore, it most likely constituted sporadic low-level contamination (data not shown).

DNA was extracted from a total of 147 samples in 13 batches using the modified SF method (Table 3). The extraction was repeated using a 10 fold amount of bone powder and the PCI method for 43 samples that showed low DNA yields in the first round (cut-off: <2 pg/μl). This brought higher DNA yields in nine samples (Table 4). In the remaining 34 samples the repeated extraction with the PCI method did not result in higher DNA quantities (data not shown). Altogether, the extraction experiments resulted in a total of 141 samples with detectable DNA yields (>0.24 pg/μl; Table 5, Fig. S3).

3.3. Molecular genetic sex-typing

Molecular genetic sex-typing was performed using the PCR multiplex Genderplex [3]. This assay includes two differently sized amelogenin targets (AMELX/Y short: 55/58 bp; AMELX/Y long: 106/112 bp), one SRY target (93 bp) and four X-chromosomal STRs also featuring short amplicon lengths. This tool has proven very

Table 2
Summary of the quantification results from the extraction pre-studies. SC – silica columns [7]; PCI – phenol/chloroform/isoamyl-alcohol [8]; SF – spin filter [9]; n.d. – none detected.

Method	First pre-study – yield of nuclear DNA (pg/μl)						Second pre-study – yield of nuclear DNA (pg/μl)							Contamination	Handling	Toxic
	V1	Blank	V2	Blank	Kals	Blank	Ind 1 (tooth)	Ind 1 (bone)	Ind 2 (tooth)	Ind 3 (bone 1)	Ind 3 (bone 2)	Ind 3 (tooth)	Blank			
SC	0.5	0.5	7.9	0.1	161.6	3.1	–	–	–	–	–	–	–	Yes	Complex	+/–
PCI	0.4	n.d.	6.7	0	77.2	n.d.	15.4	376.2	3.3	8.0	0.8	22.5	0.1	No/minor	Complex	+
SF	0.7	n.d.	5.2	0	0.2	n.d.	24.0	44.2	6.2	8.6	1.4	64.3	0.3	No/minor	Easy	–

Table 3
Summary of extraction dates and sample numbers of the main study and quantification results of corresponding extraction blanks. RFU – relative fluorescence units.

Extraction	Extraction method	Number of samples	gDNA in blanks (pg/μl)	Amel	D3S1358	TH01	D21S11	D18S51	D10S1248	D16S539	vWA	D8S1179	FGA	D2S441	D12S391	D19S433	Sex typing Genderplex	Amel long
Pre-study 1	SC	3	3.10	X (380 RFU)		9.3 (400 RFU)	30.2 (200 RFU) 30.2 (60 RFU)		15 (300 RFU)				23 (250 RFU)		18 (200 RFU)	14 (1000 RFU)		
	PCI	3	n.d.															
	SF	3	n.d.															
Pre-study 2	PCI	3	0.10							9 (70 RFU)		13 (50 RFU)				12 (250 RFU)	No profile	
	SF	3	0.30															
05/27/10	PCI	4	0.04															
11/11/10	SF	7	1.60	X, Y (150 RFU)		6 (120 RFU)		16 (100 RFU)		11 (120 RFU)	18 (100 RFU)					13 (80 RFU)	No profile	
12/23/10	SF	7	0.06														No profile	
02/01/11	SF	11	0.14														No profile	
04/01/11	SF	11	0.18						15 (300 RFU)								No profile	
04/12/11	SF	11	0.17														No profile	
04/20/11	SF	11	0.14														No profile	
07/14/11	SF	11	0.03														No profile	
01/13/12	SF	13	0.06														No profile	
02/10/12	SF	13	0.02														No profile	
02/23/12	SF	13	0.04														No profile	
02/29/12	SF	13	0.03														No profile	
03/03/12	SF	13	0.06														No profile	
03/07/12	SF	13	0.25													14 (400 RFU)	No profile	
04/04/12	PCI	11	0.03														No profile	
04/05/12	PCI	11	0.08						13 (350 RFU)					14 (150 RFU)			No profile	
04/19/12	PCI	11	0.07		18 (200 RFU)												No profile	
04/25/12	PCI	10	0.01						13 (300 RFU)								Partial profile	X (100 RFU)

Total extract volume: 50–75 μl.

Table 4
Nine out of 43 samples showed higher gDNA yields after repeated extraction using the PCI protocol with higher amounts of bone powder in the lysis process.

Grave no.	Extraction method	gDNA (pg/μl)
34	SF	0.7
	PCI	4.6
43	SF	1.9
	PCI	48.5
74	SF	1.2
	PCI	7.9
76	SF	1.5
	PCI	6.5
81	SF	0.8
	PCI	2.9
101	SF	0.1
	PCI	6.3
106	SF	0.5
	PCI	27.0
119	SF	1.5
	PCI	21.4
143	SF	1.1
	PCI	14.3

useful to unambiguously determine the sex status of a sample due to the parallel testing of different regions on both gonosomes. The short amelogenin X/Y amplicons are particularly suitable for the analysis of heavily degraded DNA. For less degraded as well as high quality DNA the two amelogenin genotypes should match to allow for a reliable sex-typing result. In addition, samples from male individuals are expected to display an SRY peak, while samples from females usually result in multiple heterozygous X-STR genotypes (Fig. S3, [3]).

The DNA extracts from this study were sorted by descending DNA quantity and subjected to Genderplex analysis. The experiments were stopped after the analysis of 107 samples, as no detectable amplification products were obtained beyond the 96th sample. Eighty-eight of these samples yielded interpretable Genderplex profiles (Table S1), while the remaining eight samples, all having very low gDNA contents, had to be excluded from the data set as they showed discrepant amelogenin results. Taken together, we found 35 female and 53 male genotypes.

3.4. Comparison to morphological sex typing

In the course of anthropological examinations in 2001 standard osteological data was collected on the remains from the mediaeval cemetery. Individual sex was determined using standard morphological methods [1,15]. In some cases a poor level of bone preservation or loss of certain skeletal elements required for sexing did not allow a sex diagnosis. As a result of this study, both morphological and molecular genetic sex-typing results for 78 samples were available. In 21 cases (27%) the results of the two different methodological approaches were discordant. In the course of this study, performed almost 10 years after the initial morphological sex identification attempts, a morphological review of the discrepant samples was performed. Twenty of the 21 skeletons were investigated, only the remains found in Burial 125 were not available. Due to the different kind, age and burial conditions of the remains the following categories were established after the re-examination: (i) morphological data completely or by trend confirming the genetic data ($n = 8$); (ii) morphological data unsecure but by trend confirming genetic results ($n = 2$); (iii) morphological results ambiguous but by trend in disagreement with genetic data ($n = 2$); (iv) morphological results were clear and remained in disagreement with genetic data ($n = 3$) and (v) available remains were not fit for morphological sex identification ($n = 5$). The reasons for the remaining discordant sex-typing results were difficult to determine. The genetic data seemed reliable as those samples were repeatedly typed with the Genderplex assay and yielded confirmable results, which is why human error in the laboratory process or technical errors could be excluded. Further genetic testing using autosomal and Y-chromosomal STRs also confirmed the Genderplex results in all but one sample (Burial 33, Table S1). There, no Y-STR profile was observed in the DNA extract that revealed a male-specific Genderplex profile but was morphologically sex-typed as female. DNA contamination was unlikely but could not be fully excluded. When performed on a complete skeleton in which sex-related bone features such as the pelvis are present, the method of morphological sex identification can have a success rate up to 95% and error of 2% [15]. Unfortunately, the pelvic bones, especially the pubic region, are often damaged due to their fragility. This was also the case in the Volders series. Modern disturbance of graves resulted in destruction of certain portions of the skeleton in a number of burial situations. Of the 21 individuals for which a discrepancy in sex identification between the two methods was observed, 11 were incompletely preserved (i.e. only a handful of bone fragments or parts of the skeleton such as the lower extremities were available for examination) and one was too poorly preserved to allow for accurate sex identification. Of these 11 partial skeletons, 5 had no sex-related features at all and the choice of a tendency towards male or female was based alone on characteristics such as bone robustness and size. In two further cases there was clear evidence for commingling of skeletal elements from more than one grave, which may have resulted over the years in the course of handling, storage and repeated transportation. Two other skeletons belonged to young juveniles. Sexing juvenile skeletons is complicated by the lack of matured secondary sexual characteristics and generally limited to a diagnosis that suggests a tendency to male or female. Only in 4 cases was the morphologically based sexing thought to be accurate with certainty, since sex-related features were present and assessable. As mentioned previously, one skeleton could not be located and was therefore no longer available for the follow-up examination.

Table 5
Summary of the extraction experiments of the main study. A total of 141 specimens yielded detectable gDNA concentrations.

pg/μl gDNA	# Samples
0.24–4	40
5–10	21
11–50	45
51–100	17
101–1695	18

Our study conclusively demonstrates that reliable sex-typing with redundant genetic targets provides a useful tool for archaeological and forensic applications, particularly in situations involving poor or incomplete skeletal preservation. The importance of providing accurate sex identifications for large skeletal series has an impact on all subsequent demographic examinations and their implications. Because morphologically based examinations are significantly influenced by skeletal preservation, many demographic studies of archaeological skeletal series are based on incomplete data. Combining the sexing results gathered from genetically and morphologically based methods will certainly lead to more complete data sets, thereby increasing the integrity of population demographics.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2013.05.005.

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